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Enhanced production of pediocin PA-1 in wild nisin- and non-nisin-producing *Lactococcus lactis* strains of dairy origin

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Abstract

In this work, heterologous production of pediocin PA-1 in *Lactococcus lactis* ESI 153 and ESI 515 (Nis$^+$), two strains selected because of their technological properties for cheesemaking, was achieved after transformation with plasmids pMC117, pRK119 and pCNC1, which contain the complete pediocin operon under the control of the strong P32 promoter. The pediocin production of the *L. lactis* ESI 153 derivatives containing pRK119 or pCNC1 was higher (approximately 165%) than that achieved by the natural pediocin PA-1 producer *Pediococcus acidilactici* 347. In the case of the *L. lactis* ESI 515 derivatives, those containing pRK119 or pCNC1 showed a pediocin production level similar (95–100%) to that of *P. acidilactici* 347.

Keywords: *Lactococcus lactis*; Pediocin PA-1; Nisin; Heterologous production

1. Introduction

The bacteriocin pediocin PA-1 is interesting as a food biopreservative because of its strong activity against *Listeria monocytogenes* (Muriana, 1996), a major biological hazard in the dairy industry. Therefore, production of pediocin PA-1 in strains of dairy origin is an attractive objective. Heterologous production of this bacteriocin by strains of *Lactococcus lactis* has been achieved using two strategies: introduction of the whole pediocin PA-1 operon (*pedABCD*) (Buyong, Kok, & Luchansky, 1998; Chikindas, Venema, Ledeboer, Venema, & Kok, 1995), or the exploitation of the amino acid homologies shared among leader peptides, and also among transporters of most class II bacteriocins (Horn et al., 1998; Horn et al., 1999; Horn, Fernández, Dodd, Gasson, & Rodríguez, 2004; Reviriego et al., 2005). However, the results of the studies cited above cannot be compared because of the different hosts, plasmids, promoters, substrates and antimicrobial assays employed.

In a previous study (Reviriego et al., 2005), pediocin-producing derivatives of the starter strains *L. lactis* ESI 153 and ESI 515 (CL1 and CL2, respectively) were constructed by introduction of pFI2160. This pTG262-derivative vector includes a fragment (L*ped-lcnCD*) that contains a hybrid gene encoding the lactococcin A leader fused to the mature part of pediocin PA-1, and the genes *lcnC* and *lcnD* that specify the lactococcin A secretion apparatus (Horn et al., 1999). The objective of this work was to elucidate if pediocin production could be enhanced in such hosts after transformation with plasmids harbouring the *ped* operon under the control of a stronger promoter. In addition, we investigated if chromosomal integration of the *ped* genes could be an alternative for pediocin PA-1 production in lactococcal hosts.

2. Materials and methods

2.1. Microorganisms and culture conditions

The lactococcal strains used in this study are listed in Table 1. *L. lactis* ESI 153 and ESI 515 (a nisin-producing strain; Nis$^+$) were originally isolated from artisan raw milk

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Table 1
Lactococcal strains used in this study

Strain	Host	Plasmid	Reference or source
MG1614			Gasson (1983)
LL108 (*repA*$^+$)	MG1363		Leenhouts et al. (1998)
LL108O	LL108	pORI280-GalA'(Ery^R, pORI280 derivative, contains a *Bam*HI site between the initial and the final sequence of the *galA* gene of *L. lactis*)	W.A. Pool and A.R. Neves
LL108W	LL108	pWRC03 (Ery^R, pORI280-GalA' derivative, contains the hybrid *L-pedA* gene preceded by the lactococcin A promoter, *lcnC* and *lcnD*)	This study
IL1403MC	IL1403	pMC117 (Ery^R, pMG36e derivative, contains the pediocin operon preceded by the P32 promoter)	Chikindas, Venema, Ledeboer, Venema, and Kok (1995)
MG1363RK	MG1363	pRK119 (Cm^R, pMG36c derivative, contains the pediocin operon preceded by the P32 promoter)	R. Kemperman
MG1614CNC	MG1614	pCNC16 (Cm^R, pTG262 derivative, contains the pediocin operon preceded by the P32 promoter)	This study
CR	MG1614	Plasmid free; the pWRC03 fragment containing *L-pedA*, *lcnC* and *lcnD* has been integrated in the chromosome	This study
ESI 153			Cogan et al. (1997)
CL1	ESI 153	pFI2160 (Cm^R, pTG262 derivative, contains the hybrid *L-pedA* gene preceded by the lactococcin A promoter, *lcnC* and *lcnD*)	Reviriego et al. (2005)
MC1	ESI 153	pMC117	This study
RK1	ESI 153	PRK119	This study
CNC1	ESI 153	pCNC16	This study
ESI 515 (Nis$^+$)			Cogan et al. (1997)
CL2	ESI 515	pFI2160	Reviriego et al. (2005)
MC2	ESI 515	pMC117	This study
RK2	ESI 515	PRK119	This study
CNC2	ESI 515	pCNC16	This study

cheeses (Cogan et al., 1997), and have been successfully used as adjuncts and/or starter cultures in cheese manufacture (Rodríguez, Gaya, Nuñez, & Medina, 1998). Lactococcal strains were grown in M17 (Oxoid, Basingstoke, UK) supplemented with 0.5% (w/v) glucose (GM17 medium) or MRS (Oxoid) while *Pediococcus acidilactici* 347 (Rodríguez et al., 1997) was grown in MRS. Cultures were incubated at 32 °C without agitation. When appropriate, chloramphenicol (Cm; Sigma, St Louis, USA) or erythromycin (Ery; Sigma) was added (5 μg mL^{-1}) to the cultures.

2.2. Construction of lactococcal strains

Three plasmids harbouring the complete *ped* operon preceded by the P32 promoter (Table 1) were used to elucidate if pediocin production could be enhanced in ESI 153 and ESI 515: (1) pMC117, a pMG36e derivative (Chikindas et al., 1995); (2) pRK119, a pMG36c derivative kindly provided by R. Kemperman (University of Groningen, The Netherlands); and (3) pCNC16, a pTG262 derivative that was constructed as follows. A DNA fragment (3659-bp) containing the pediocin PA-1 operon under the control of the P32 promoter was obtained by PCR from pRK119 using primers operonF (5′-CGAAGATCTGATATGATAAGATTAATAGTT-3′) and operonR (5′-CGAAGATCTCTATTCTTGATTATGAATTAAC-3′), which include *Bgl*II sites (underlined), and the Platinum *Taq* DNA polymerase (Invitrogen, Paisley, UK). PCR conditions have been described previously (Reviriego et al., 2005). After digestion with *Bgl*II (New England Biolabs, Ipswich, USA), the fragment was cloned in the *Bam*HI site of pTG262 generating pCNC16.

Transformation of the lactococcal hosts with pCNC16, pMC117 and pRK119 as described by Wells, Wilson, and Le Page (1993) generated strains MC1, RK1 and CNC1, respectively, in the case of *L. lactis* ESI 153, and strains MC2, RK2 and CNC2, respectively, in that of ESI 515. Pediocin PA-1 and nisin antimicrobial activities were assayed by a diffusion bioassay with *Enterococcus faecalis* TAB28 (pediocin-sensitive; Joosten, Rodríguez, & Nuñez, 1997) and *L. lactis* MG1614 (nisin-sensitive; Gasson, 1983) as indicator organisms. The supernatants were obtained from cultures with a bacterial concentration of 1×10^9 cfu mL^{-1}. *L. lactis* BB24 (Nis$^+$; Rodríguez et al., 1995) and *P. acidilactici* 347 (Ped$^+$) were used as positive controls.

Pediocin PA-1 activity in the culture supernatants was quantified by a microtiter plate assay system (Holo, Nilssen, & Nes, 1991) with *E. faecalis* TAB28 as indicator. The assays were performed in quadruplicate and the standard deviations (SD) values were calculated.

2.3. Chromosomal integration of the genes required for pediocin PA-1 biosynthesis in L. lactis MG1614

The fragment L*ped-lcnCD* was amplified from pFI2160 (Horn et al., 1999) with primers F*Bgl*IIlcnC (5′-CGAAGATCTGAGGCAGTAAGTAATATTATTTTC-3′) and

R*Bgl*IIhybr (5′-CGAAGATCTGCATAATGCTAGCATT-TATGAT-3′) (annealing at 50 °C), which include *Bgl*II sites (underlined). The resulting 4099-bp fragment was cut with *Bgl*II and cloned in the *Bam*HI site of the non-replicative vector pORI280-GalA′ (Table 1) to generate pWRC03. This plasmid was introduced into *L. lactis* LL108 for maintenance purposes since this strain carries several copies of *repA* in its chromosome (Leenhouts, Bolhuis, Venema, & Kok, 1998); then, pWRC03 was introduced into *L. lactis* MG1614, a strain in which the plasmid can not replicate, enabling the integration of the genes required for pediocin PA-1 production into its chromosome. Integration of the fragment L*ped-lcnCD* (4093 bp) in the chromosome of *L. lactis* MG1614 was assessed by PCR amplification using the primers FlcnC (5′-GAGGCAGTAAGTAATATTATTTTC-3′) and Rhybr (5′-GCATAATGCTAGCATTTATGAT-3′) (annealing at 50 °C).

3. Results and discussion

Pediocin production was detected in the culture supernatants of strains CL1, MC1, RK1, CNC1, CL2 MC2, RK2 and CNC2 (Fig. 1a). The smallest inhibition zones corresponded to CL1 and CL2 supernatants while those obtained by pRK119- or pCNC16-containing strains (RK1 and RK2 or CNC2 and CNC1, respectively) displayed the largest halos (Fig. 1a). These results were in agreement with those obtained with the microtitre plate assay (Table 2). Pediocin production by CL1 and CL2 (Table 1) had already been achieved through the leader/transporter exchange strategy (Reviriego et al., 2005). However, the pediocin activity observed in those cultures was notably lower than that displayed by the strains carrying the *ped* operon constructed in this study (Fig. 1a; Table 2). This may be a consequence of a higher biosynthetic capacity of strains containing the complete pediocin operon and/or the effect of the strong P32 promoter since in pFI2160 the genes required for pediocin biosynthesis are under the control of the lactococcin A promoter. The highest pediocin production was achieved by strains RK1 and CNC1, which displayed 163% and 165%, respectively, of the activity displayed by *P. acidilactici* 347 (Table 2). The pediocin activity showed by *L. lactis* MC1 (pMC117), was more than ten times lower than that detected in RK1 (pRK119) supernatants (Table 2). Curiously, the main difference between pMC117 and pRK119 is the antibiotic resistance marker (Ery and Cm, respectively).

The presence in *L. lactis* ESI 515 of either pMC117, pRK119 or pCNC16 did not alter its nisin-producing phenotype (Fig. 1b), a finding that has been previously reported when this strain has been used as a host for pediocin PA-1 production (Horn et al., 1999, 2004; Reviriego et al., 2005). Pediocin production by strain MC2 (pMC117; 751 BU) was three times higher than in *L. lactis* MC1 (pMC117; 225 BU). In contrast, pediocin

Table 2
Pediocin PA-1 production by the strains used in this study

Strain	Pediocin PA-1 activity (BU ± SD)	%
P. acidilactici 347	1500 ± 29	100
L. lactis ESI 153	Nd[a]	—
L. lactis CL1	106 ± 12	7
L. lactis MC1	225 ± 15	15
L. lactis RK1	2447 ± 43	163
L. lactis CNC1	2478 ± 39	165
L. lactis ESI 515	Nd	—
L. lactis CL2	33 ± 7	2
L. lactis MC2	751 ± 22	50
L. lactis RK2	1503 ± 34	100
L. lactis CNC2	1427 ± 40	95
L. lactis CR	Nd	—

[a]Nd, not detected.

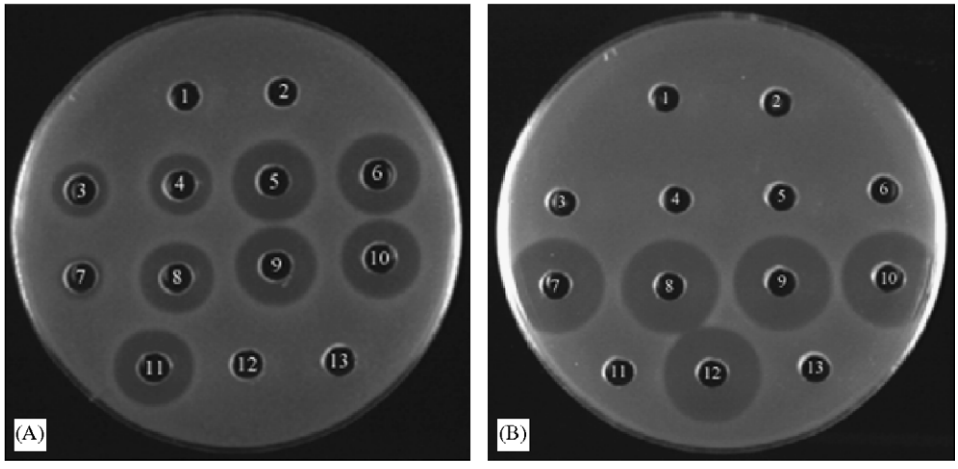

Fig. 1. Agar diffusion bioassay for detection of pediocin PA-1 activity against *Enterococcus faecalis* TAB28 (A) and nisin activity against *L. lactis* MG1614 (B). Samples: 1 and 2, *L. lactis* ESI 153; 3, *L. lactis* CL1; 4, *L. lactis* MC1; 5, *L. lactis* RK1; 6, *L. lactis* CNC1; 7, *L. lactis* CL2; 8, *L. lactis* MC2; 9, *L. lactis* RK2; 3, *L. lactis* CNC2; 11, *P. acidilactici* 347; 12, *L. lactis* BB24; 13, *L. lactis* MG1614.

activity in the supernatants of the ESI 515-derivatives (Nis^+) carrying pRK119 (1503 BU) or pCNC16 (1427 BU) was lower than that found in the corresponding ESI 153-derivatives (Nis^-) (2447 and 2478 BU, respectively) (Fig. 1; Table 2). A reduced pediocin production in nisin/pediocin co-producing lactococci strains, in comparison with non-nisin producing *L. lactis* strains, has been previously reported (Horn et al., 1999, 2004; Reviriego et al., 2005). Nevertheless, the level of pediocin production by *L. lactis* RK2 and CNC2 was approximately that observed in the wild strain *P. acidilactici* 347 (Fig. 1; Table 2). To our knowledge, this represents the first report of *L. lactis* strains technologically suited for cheese making with the ability to co-produce pediocin PA-1 and nisin at levels comparable to the respective parental strains (Table 2).

Finally, a DNA fragment carrying the genes required for production of pediocin PA-1 was successfully integrated in the chromosome of *L. lactis* MG1614. However, production of pediocin PA-1 could not be detected in the supernatants of the integrant, probably because of the sharp reduction in the copy number of the *ped* genes (1 per cell). Work is in progress to obtain pediocin-producing *L. lactis* ESI 153 and ESI 515 derivatives suited for dairy applications by the development of food-grade vectors.

4. Conclusions

L. lactis ESI 153 and ESI 515 derivatives carrying P32-*ped* operon-containing plasmids produced more pediocin PA-1 than *L. lactis* CL1 and CL2, in which a strategy based on leader/transporter exchange was used. The enhanced production of pediocin PA-1 in *L. lactis* RK1 and CNC1, and the high coproduction of pediocin PA-1 and nisin in *L. lactis* RK2 and CNC2, makes these new strains very attractive for the control of *L. monocytogenes* and other pathogens in dairy products.

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